1

National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service

Services des thèses canadiennes

Ottawa, Canada
K1A 0N4

CANADIAN THESES

THÈSES CANADIENNES

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

**THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED**

**LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE**

National Library
of Canada

Bibliothèque nationale
du Canada

0-315-22160-7

Canadian Theses Division Division des thèses canadiennes

Ottawa, Canada
K1A 0N4

PERMISSION TO MICROFILM — AUTORISATION DE MICROFILMER

- Please print or type — Écrire en lettres moulées ou dactylographier

Full Name of Author — Nom complet de l'auteur

Roy Dwight COLE

Date of Birth — Date de naissance

August 1, 1960

Country of Birth — Lieu de naissance

Canada

Permanent Address — Résidence fixe

140-790 Springland Drive
Ottawa, Ontario
K1V 6L7

Title of Thesis — Titre de la thèse

Temperature Effect on Bivalve Anaerobic Metabolism

University — Université

Carleton

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

M.Sc.

Year this degree conferred — Année d'obtention de ce grade

1984

Name of Supervisor — Nom du directeur de thèse

Dr. K.B. Storey

Permission is hereby granted to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

L'autorisation est, par la présente, accordée à la BIBLIOTHÈQUE NATIONALE DU CANADA de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans l'autorisation écrite de l'auteur.

Date

9/10/84

Signature

Roy Cole

TEMPERATURE EFFECT ON
BIVALVE ANAEROBIC METABOLISM

by

ROY DWIGHT COLE, B.Sc. (Hons. Biology)

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfilment
of the requirements for the degree of
Master of Science

Department of Biology
Carleton University
Ottawa, Ontario.

September 4, 1984

© copyright

1984, Roy Cole

LICENCE TO CARLETON UNIVERSITY

In the interests of facilitating research by others at this institution and elsewhere, I hereby grant a licence to:

CARLETON UNIVERSITY

to make copies of my thesis:

Temperature Effect on Bivalve Anaerobic Metabolism

or substantial parts thereof, the copyright which is invested in me, provided that the licence is subject to the following conditions:

Only singled copies shall be made or authorized to be made at any one time, and only in response to a written request from the library of any university or similar institution on its own behalf or on behalf of one of its users.

This licence shall continue for the full term of the copyright, or for so long as may be legally permitted.

The Universal Copyright Notice shall appear on the title page of all copies made under the authority of this licence.

This licence does not permit the sale of authorized copies at a profit, but does permit the collection by the institution or institutions concerned of charges covering actual costs.

All copies under the authority of this licence shall bear a statement to the effect that the copy in question "is being made available in this form by the authority of the copyright owner solely for the purpose of private study and research and may not be copied or reproduced except as permitted by the copyright laws without written authority from the copyright owner."

The foregoing shall in no way preclude the granting by the author of a licence to the National Library of Canada to reproduce the thesis and to lend or to sell copies of the same. For this purpose it shall also be permissible for Carleton University to submit the abovementioned thesis to the National Library of Canada.

Kew Stirling
Signature of Witness

9/10/84

Date

M.Sc.
Degree

Roy Cole
Signature of Student

Biology
Department of Student

Abstract

The response of anaerobic metabolism in Mercenaria mercenaria to long term and acute temperature stress revealed qualitative and quantitative changes in metabolic flux.

- a) The rate of anaerobic metabolism was sensitive to both long term and acute temperature change.
- b) The choice of anaerobic end product was affected by environmental temperature
- c) Long term exposure to high temperature results in acclimation of anaerobic metabolism not seen during acute exposure to high temperature.

The undersigned hereby recommend to the Faculty of
Graduate Studies and Research acceptance of this thesis,
submitted by Roy Dwight Cole, B.Sc., in partial fulfillment
of the requirements for the degree of Master of Science

.....
Chairman, Department of Biology

.....
Supervisor

Carleton University

Acknowledgements

A work of this complexity is not possible without the help and support of many people. Firstly I wish to thank Dr. Ken Storey for his encouragement and guidance. Also the technical assistance and advice of Jan Storey and my fellow lab workers. I would like to express my gratitude to my advisors, Dr. D. Gardener and Dr. K. Joy for their help. Most of all I want to thank April whose love and hard work has supported me in this project.

The research was supported by an NSERC postgraduate scholarship.

Table of Contents

	Page No.
Abstract	iii
Acknowledgement	iv
Table of Contents	v
List of Tables	vi
List of Figures	vii
List of Abbreviations	ix
Introduction	1
Materials and Methods	18
Results	28
Discussion	71
References	84

List of Tables

	Page No.
Table 1 Levels of Protein, Glycogen and Lipid in tissues of Control <u>M. mercenaria</u> .	30
Table 2 Changes in the tissue levels of metabolites, 96h minus 0h, during anoxia.	39
Table 3 Levels of alternative anaerobic end products in the tissues of <u>M. mercenaria</u> after 96h of anoxia.	50
Table 4 Glucose levels in tissues and blood samples of <u>M. mercenaria</u> (long term temperature study).	51
Table 5 Tissue glucose levels during anoxia and recovery (acute temperature study).	54
Table 6 Q_{10} values for accumulation of succinate and use of glycogen in the tissues of <u>M. mercenaria</u> after 96h of anoxia.	75

List of Figures

	Page No.
Figure 1 Scheme for anaerobic energy metabolism in bivalve molluscs. (Zurburg, 1981)	6
Figure 2 Changes in carbon flow of bivalve molluscs under conditions of A. aerobiosis, B. early anaerobiosis, C. late anaerobiosis, D. reimmersion.	13
Figure 3 The changes in concentration of succinate, alanine, aspartate, and glycogen in the mantle of <u>M. mercenaria</u> (long term study).	34
Figure 4 The changes in concentration of succinate, alanine, aspartate, and glycogen in the gill of <u>M. mercenaria</u> (long term study)	36
Figure 5 The changes in concentration of succinate, alanine, aspartate, and glycogen in the adductor of <u>M. mercenaria</u> (long term study).	38
Figure 6 The changes in concentration of succinate, alanine, aspartate, and glycogen in the mantle of <u>M. mercenaria</u> (acute study).	66

- Figure 7 The changes in concentration of succinate, 68
alanine, aspartate, and glycogen in the
gill of M. mercenaria (acute study).
- Figure 8 The changes in concentration of succinate, 70
alanine, aspartate, and glycogen in the
adductor of M. mercenaria (acute study).

List of Abbreviations and Terms

AMP	-adenosine monophosphate
ATP	-adenosine triphosphate
°C	-degrees centigrade
g.w.w.	-gram wet weight
ITP	-inosine triphosphate
μl	-microlitre
μM	-micromolar
ml	-millilitre
mM	-millimolar
mm Hg	-millimetres mercury
mOsm	-milliosmolar
nm	-nanometre
NAD	-nicotinamide adenine dinucleotide
NADH	-nicotinamide adenine dinucleotide reduced
N	-normal
PCA	-perchloric acid
PO ₂	-partial pressure of oxygen
PEP	-phosphoenolpyruvate
PEPCK	-phosphoenolpyruvate carboxykinase
PK	-pyruvate kinase
Q ₁₀	<u>-change of metabolite at T° + 10</u> change of metabolite at T°
Tris	-tris (hydroxymethyl) aminomethane
U	-units

INTRODUCTION

(de Zwaan et al., 1983). Tissue specific differences in anaerobic metabolism are also reflected in the enzyme profile of various species. PEPCK activity has been found to vary widely between different tissues (de Zwaan, 1983).

Temperature change, like limitation in oxygen availability, is an inescapable environmental stress of the intertidal environment. Temperature fluctuations must be faced over both the short term, daily cycles of submergence and aerial exposure and over the long term with changing seasons. The effects of temperature on the metabolism of ectotherms have long been an area of intense research (Bullock, 1955; Precht, 1958; Prosser, 1955). This work has produced the widely accepted theory that the biological activities of ectotherms change by a factor of two for every 10°C change in temperature ($Q_{10}=2$) within the physiological temperature range. This includes not only metabolic rate but also other physiological activities such as digestion and locomotion. The effects of temperature on metabolic rate could have a major impact on the ability to survive in the intertidal environment, particularly under anaerobic conditions.

Recent studies of intertidal molluscs have suggested that this group of animals is able to maintain a metabolic rate independent of temperature (Newell and Pye, 1971; Newell, 1969; Shirley et al., 1978; Widdows and Bayne, 1971). An important aspect of this temperature independence

5

Anaerobic metabolism in vertebrates is well studied and consists of the fermentation of carbohydrates, glycogen or glucose, via the reactions of glycolysis to form lactic acid. This pathway is typically used for short term energy production when energy demand exceeds the oxidative capacity of the tissue; an example of this is burst muscular work. Such an energy demand induces a rapid increase in the rate of cytoplasmic glycolysis and carbohydrate consumption. All of the catabolized glucose carbon is channelled into the production of lactic acid with a net production of 3 ATP molecules per glucosyl unit from glycogen; the final reduction step, to lactate, serves in maintaining the cytoplasmic redox balance. For most vertebrates this is a short term response only and relies upon a rapid return to the aerobic condition followed by the repayment of an oxygen debt. However, intertidal animals must meet different goals during anaerobiosis. The problem which they face is not short term energy production, rather it is the survival of long term anoxia.

Survival of anoxia during aerial exposure of intertidal bivalves depends on two types of adaptations 1) a dramatic decrease in energy utilization and 2) use of altered metabolic pathways which increase the efficiency of anaerobic energy production. The obvious physical activities of nonsessile bivalves (example: digging, filter feeding) cease during aerial exposure; this suggests a reduced

energy demand. This concept was supported by a calorimetric study of bivalve anoxia (Shick et al., 1983), which showed anaerobic heat production to be less than 10% of the aerobic level. However even more important to bivalve survival of anoxia has been the restructuring of the metabolic pathways.

Similar to the vertebrate situation, glycogen is the major fuel of anaerobic metabolism in the bivalves and it is catabolized by the glycolytic pathway. Lactic acid, however, is not the major end product (Zandee et al., 1980a). Instead, a variety of possible end products are found including alanine, succinate, malate, glutamate, the volatile fatty acids propionate and acetate, and the imino acids strombine and alanopine (Fig. 1.). The use of radiolabelled glucose has shown that most carbon is catabolized to either alanine or succinate (Collicutt and Hochachka, 1977; Stokes and Awarpa, 1968). The amino acid aspartate is an additional substrate of anaerobic energy metabolism (Collicutt and Hochachka, 1977). A key difference to be noted, as opposed to the vertebrate situation, is the involvement of both cytoplasmic and mitochondrial pathways in the formation of anaerobic end products in bivalves. This is a more sophisticated metabolism than that of vertebrate glycolysis and results in a more efficient production of ATP per glycosyl unit utilized.

The bivalve catabolism of glycogen to phosphoenolpyruvate (PEP) follows the classical glycolytic pathway. However

Figure 1 Scheme for anaerobic energy metabolism in bivalve molluscs (Zurburg, 1981).

Abbreviations: AC - acetate; ALA - alanine; ALN - alanopine; ASP - aspartate; LAC - lactate; OCT - octopine; PROP - propionate; Str - strombine; SUC - succinate; Fru-6P - fructose-6-phosphate; Fru-1,6P - fructose-1,6-diphosphate; triose-P - triose phosphate; 1,3DPG - 1,3 diphosphate glycerol; 3PG - 3-phosphoglycerol; PEP - phosphoenolpyruvate; Glu - glutamate; 2-OG - α -ketoglutarate; OAA - oxaloacetate; MAL - malate; Fum - fumarate; ACCoA - acetyl-Coenzyme A; MMCoA - methylmalonyl-Coenzyme A; PROPCoA - propionyl-Coenzyme A; SUCCoA - succinyl-Coenzyme A.

at the level of PEP there are two possible routes which can be followed (Fig. 1). Pyruvate kinase may act to catalyse the conversion of PEP to pyruvate, with the formation of one ATP molecule. Under aerobic conditions pyruvate would then be oxidized by the Krebs cycle. During anoxia, however, pyruvate can be converted into one of several organic end products. Transamination of pyruvate results in the formation of alanine, this reaction involving the transfer of an amino group from a donor molecule to pyruvate; most often the donor molecule is the substrate aspartate. An alternative fate for pyruvate is condensation with an amino acid to form alanopine or strombine by one of the following reactions:

If there are no other pathways in operation these reactions have the advantage of being catalyzed by dehydrogenase enzymes. This regenerates the NAD consumed at the glyceraldehyde-3-phosphate dehydrogenase reaction thus maintaining cytoplasmic redox balance.

The reactions leading to succinate or volatile fatty acid production are the truly innovative portions of bivalve anaerobic metabolism for they result in the synthesis of additional ATP molecules. There are two possible pathways

by which carbon enters into succinate formation. The carbon skeleton of aspartate can be utilized after transamination with the resulting oxaloacetate converted to succinate by a reversal of several Krebs cycle reactions. Formation of ATP occurs at the fumarate reductase step (Zurburg, 1981).

Alternatively succinate can also be formed from glycolytic carbon by channelling PEP through the PEP carboxykinase (PEPCK) reaction rather than through pyruvate kinase (PK). The reaction catalyzed by PEPCK is the carboxylation of PEP to oxaloacetate. Similar to PK the PEPCK reaction results in the formation of a high energy molecule, ITP. Oxaloacetate is then reduced to malate by the cytoplasmic malate dehydrogenase reaction. This step regenerates NAD to maintain cytoplasmic redox balance in the absence of pyruvate oxidoreductase reactions. Malate crosses the mitochondrial membrane and enters the Krebs cycle where it is converted to succinate by the reversal of two Krebs cycle reactions. This pathway is more energy efficient than the conversion of PEP to alanine due to the ATP formed at the fumarate reductase reaction. In some bivalve species the efficiency of the succinate path is further enhanced by the conversion of succinate to propionate resulting in additional ATP synthesis (de Zwaan, 1977).

The control over the partitioning of carbon between the PEPCK versus PK reactions (the so called "PEP branchpoint")

has received extensive attention. Early studies of the PEP branchpoint identified two factors which could inhibit PK activity and thus promote PEPCK activity during anoxia. One of these factors is the change in the pH of the body fluids during anoxia. In the early minutes of shell closure the pH of the mantle fluid drops (Crenshaw and Neff, 1969; Wijsman, 1975), due to the increase in CO_2 levels and the accumulation of acidic anaerobic end products. Kinetic studies of PK have shown that the enzyme has an alkaline pH optima while PEPCK has a much more acidic optimum (Hochachka and Mustafa, 1972); therefore the drop in pH during anoxia would favor carbon flow through PEPCK. The second factor involved is the accumulation in the early stages of anoxia of alanine, an inhibitor of pyruvate kinase. It has been proposed by Hochachka (1973) that the change in alanine levels and pH act synergistically to inactivate PK, thus switching the direction of carbon flux to succinate production via the PEPCK reaction.

Recent studies have revealed a more potent control mechanism over pyruvate kinase (Plaxton and Storey, 1984 a,b), covalent modification of enzyme activity via phosphorylation/dephosphorylation. Under anoxic conditions PK from the anoxia tolerant channelled whelk was shown to be phosphorylated. Kinetic studies of the phosphorylated enzyme revealed a much reduced substrate affinity for PEP, a reduced sensitivity to activation by fructose-1,6-diphosphate, and a stronger inhibition by alanine compared to PK from

aerobic animals. These conditions would result in a reduction of carbon flow from PK into PEPCK. Similar work on the mussel, Mytilus edulis, suggests that bivalve PK also has interconvertible variants, decreased whole animal PO_2 promoting production of a less active form (Holwerda et al., 1983).

The energy yielding fumarate reductase reaction reduces fumarate to succinate. This step requires the oxidation of NADH and poses the problem of maintaining mitochondrial redox balance. It has been suggested by de Zwaan (1983) that a small portion of malate entering the mitochondria moves along the normal route of the Krebs cycle. The pyruvate required to sustain these reactions may come from pyruvate kinase activity or from the conversion of malate to pyruvate by malic enzyme. These oxidation reactions produce the necessary NADH required for the redox balance of the fumarate reductase reaction (Fig. 1.).

Early studies of bivalve anoxia suggested that the major end products, succinate and alanine, were produced in equimolar amounts (Stokes and Awarpa, 1968). Refinements of the basic anaerobic scheme have refuted this premise and have shown tissue, species, and time dependent differences in anaerobic metabolism. The time dependent nature of end product accumulation has been considered by Zurburg and Ebberink (1981). They suggested that there are two distinct phases to anaerobic metabolism depending upon the direction of carbon flux at the PEP branchpoint (Fig. 2). During the early phase of anoxia pyruvate kinase is still

active and PEP moves along the "glucose → alanine" pathway. This leads to a rapid accumulation of alanine with a simultaneous conversion of aspartate to succinate. The second phase of anoxia is marked by the exhaustion of aspartate reserves. Pyruvate kinase is less active and PEP is consumed by PEPCK; the "glucose → succinate" path functions. Because of the two phases alanine accumulation is varied during the anoxic time course. In the early phase, anoxia is often characterized by a 1:1 ratio of alanine and succinate accumulation. However in the long term alanine accumulation declines in favor of the higher energy yield of succinate formation over the remaining time.

Propionate formation is also time dependent, produced only in long term anoxia after sufficient substrate succinate has accumulated. However, propionate synthesis is also species dependent and a study of several Mercenaria species has shown that these animals do not produce propionate (Kluytman et al., 1982) during long term anoxia.

Tissue specific differences in anaerobic metabolism have been demonstrated in M. edulis. In soft tissues, such as gill and mantle, the PEP branchpoint operates. Succinate is derived from aspartate in the short term and from glycogen in the long term. However studies of the posterior adductor muscle have shown that this tissue lacks the ability to convert PEP to succinate, all succinate being derived from the aspartate reserves.

Figure 2 Changes in carbon flow of bivalve molluscs under conditions of A) aerobiosis B) early anaerobiosis C) late anaerobiosis D) recovery (de Zwaan, 1983).

Legend:

ET - electron-transfer chain

S - malate shuttle

TF₁ - transfer of NH₂ by glutamate-oxaloacetate transferase

TF₂ - transfer of CoA by acyl-CoA transferase

(de Zwaan et al., 1983). Tissue specific differences in anaerobic metabolism are also reflected in the enzyme profile of various species. PEPCK activity has been found to vary widely between different tissues (de Zwaan, 1983).

Temperature change, like limitation in oxygen availability, is an inescapable environmental stress of the intertidal environment. Temperature fluctuations must be faced over both the short term, daily cycles of submergence and aerial exposure and over the long term with changing seasons. The effects of temperature on the metabolism of ectotherms have long been an area of intense research (Bullock, 1955; Precht, 1958; Prosser, 1955). This work has produced the widely accepted theory that the biological activities of ectotherms change by a factor of two for every 10°C change in temperature ($Q_{10}=2$) within the physiological temperature range. This includes not only metabolic rate but also other physiological activities such as digestion and locomotion. The effects of temperature on metabolic rate could have a major impact on the ability to survive in the intertidal environment, particularly under anaerobic conditions.

Recent studies of intertidal molluscs have suggested that this group of animals is able to maintain a metabolic rate independent of temperature (Newell and Pye, 1971; Newell, 1969; Shirley et al., 1978; Widdows and Bayne, 1971). An important aspect of this temperature independence

5

is the modulation of physiological activity to compensate for temperature differences. For example, in the summer upper shore mussels experience warmer temperatures than lower shore animals. The upper shore animals compensate for the increased temperature by displaying a suppressed pumping rate compared to the lower shore mussel (Newell, 1969). Also direct measurement of oxygen consumption has shown that intertidal molluscs are able to maintain oxidative metabolism independent of temperature. A study of the intertidal gastropod Littorina littorea (Newell and Pye, 1971) measured oxygen uptake in the resting and active state while varying temperature. The standard metabolic rate, while the animal was resting, was found to be insensitive over a wide range of temperature. The active metabolic rate was more sensitive to temperature change, but showed a rapid acclimation returning to the original metabolic rate. The constancy of aerobic metabolism in response to temperature change was also demonstrated in L. irrorata (Shirley et al., 1978) and in the mussel M. edulis (Widdows and Bayne, 1971; Widdows, 1976). This temperature insensitivity of metabolic rate may be a specific adaptation to intertidal life as subtidal organisms, which are not exposed to wide temperature fluctuations, do not show this ability (Newell and Pye, 1970).

Research to date has focussed on temperature and aerobic metabolic rate. However temperature extremes are perhaps more often encountered during aerial exposure when the

animal is dependent upon anaerobic metabolism. Therefore it would be meaningful to investigate the relationship of temperature and anaerobic metabolism in intertidal bivalves. This thesis presents the results of studies of the effects of temperature change (both long term acclimation and acute temperature change) on anaerobic metabolism in an intertidal clam. The experiments address the questions: 1) is the rate of anaerobic metabolism temperature dependent? 2) do the ratios of end product accumulated vary at different temperatures? 3) is the anaerobic response of animals acclimated to different temperatures the same as animals subjected to acute temperature exposure?

The subject of the present study is the cherrystone clam or quahog, Mercenaria mercenaria. This is a commercially important species along the northeastern coast of North America. It is a burrowing species found in the intertidal sand, its powerful foot allowing it to rapidly dig into the substrate. The intertidal lifestyle suggests a strong anoxic tolerance and indeed studies by Korycan and Storey (1983) have shown that individuals routinely survive 96h of anoxia. The cherrystone clam is a filter feeder of algae with a yearly cycle of reproduction that culminates with spawning in late summer. The study by Korycan and Storey (1983) demonstrated that succinate and alanine are the major anaerobic end products, while aspartate and glycogen are utilized as substrates.

The results presented here show that there are definite temperature effects of anaerobic metabolism in M. mercenaria. With respect to the three questions posed: 1) anaerobic metabolic rate is temperature dependent with respect to substrate use and end product formation 2) the ratios of end products accumulated are altered by temperature change with succinate formation favored at higher temperatures. 3) the basic effects of temperature differences on anaerobic metabolism are apparent whether temperature change results from long term acclimation or from acute temperature change.

MATERIALS AND METHODS

Animals and Chemicals

Clams, Mercenaria mercenaria, were obtained from a local seafood retailer and held in aerated, recirculating seawater tanks (100% seawater, approximately 1000 mOsmol) without feeding. All biochemicals were purchased from Sigma Chemical Co. or Boehringer Mannheim Corp.. Succinylthiokinase was a gift from Dr. W. Bridger, University of Alberta. ADH/SDH was prepared from M. mercenaria adductor muscle as described by Storey et al (1982).

Temperature Acclimation Study

Groups of clams were held in three separate tanks maintained at 25°, 15°, and 5°C. Each group was acclimated for three weeks prior to use.

Anoxia and Recovery

At the beginning of anoxia a group of control animals (n=8) were sampled directly from each seawater tank. Anoxia was imposed by placing groups of clams in jars which were flushed with nitrogen gas and sealed. The jars were held in incubators set at the temperature to which the clams had been acclimated. Clams were sampled after 2, 6, 24, and 96h of anoxia. After 96h of anoxia remaining animals were returned to aerated seawater at the respective acclimation temperature and the progress of aerobic recovery from anoxia was monitored at 6 and 24h. Eight animals were sampled at each time point during anoxia and recovery.

Five animals were rapidly opened and the gill, mantle, and adductor muscle were removed, blotted, and frozen immediately in liquid nitrogen. The other three animals were used to sample blood from the adductor sinus; samples of three animals were pooled and frozen. All tissues and blood were stored at -80°C until use.

Acute Temperature Study

All animals were in aerated seawater at 15°C for three weeks prior to use. A group of control animals ($n=5$) were sampled directly from the seawater tank at the onset of anoxia. Anoxia was again imposed by placing groups of clams in jars which were flushed with nitrogen gas and sealed. The jars were then moved to incubators at 25° , 15° , or 5°C . Anoxia clams ($n=5$) were sampled after 96h. Remaining animals were then returned to the aerated seawater at 15°C and aerobic recovery from anoxia was monitored at 24h. To test the effect of temperature shock alone, other groups of clams were transferred (simultaneously with the onset of anoxia) to aerated seawater at either 25° or 5°C . These animals ($n=5$) were sampled after 96h of exposure to each new temperature. In each instance the clams were quickly dissected and the gill, mantle, and adductor muscle were quickly removed, blotted and frozen in liquid nitrogen. The tissues were stored at -80°C prior to use.

Sample Preparation

Frozen tissue was ground to a powder under liquid nitrogen using a mortar and pestle. The powder was transferred to a preweighed, polypropylene centrifuge tube stored on dry ice. After a second weighing to determine tissue weight, tubes were transferred one at a time to a dry ice/methanol bath at -8°C . Five volumes (w/v) of 6% perchloric acid (PCA) containing 1 mM EDTA (chilled at -8°C) were quickly added and the tissue was homogenized using a Polytron PT 10 homogenizer. At this point 50 μl of homogenate was removed for glycogen determination. Tissue homogenates were centrifuged at 5900g for 10 min. at 4°C to pellet the precipitated protein. The acid supernatant was then transferred to a second tube and neutralized to approximately pH 7.0 by the addition of a solution of 3.0 N KOH/0.3 M imidazole /0.4 M KCl. After a second centrifugation to remove precipitated KClO_4 neutralized samples were stored at -80°C until analysis. In the same manner extracts of the blood samples were made (5:1, v/v) and the neutralized extracts were stored at -80°C until further analysis (Williamson and Corkey, 1969).

Glycogen Samples

The 50 μl of acid homogenate reserved for glycogen determination was added to 1 ml of acetate buffer (0.2M, pH 4.8). The pH was readjusted by the addition of 50 μl of KHCO_3 (1M). Amyloglucosidase (20 U) was added to hydrolyze the glycogen

to glucose and the solution was incubated at room temperature overnight. Samples were then stored at -20°C until analyzed for glucose (Keppler and Decker, 1974).

Aerobic Fuels

Lipid and protein levels were measured in the control animals of the temperature acclimation study.

Protein: A 100 μl sample of acid homogenate was added to 1ml of imidazole buffer (0.1M, pH7.0). The pH was readjusted by the addition of 100 μl of KHCO_3 (1M). Protein concentration was measured by the method of Bradford (1976) using the Bio-Rad prepared reagent and bovine gamma globulin as a standard.

Lipid: A portion of the frozen powdered tissue sample was set aside for the determination of glycerides. It was placed in a preweighed polypropylene centrifuge tube stored on dry ice. After a second weighing to determine tissue weight 20 volumes (w/v) of chloroform / methanol (2:1,v/v) was added to extract lipids. The tissue was homogenized and left at room temperature with periodic shaking. After 30 minutes 0.2 volumes of methanol were added to allow solid materials to sediment and samples were centrifuged at 5900g for 15 min to pellet the solids. The supernatant was removed and 0.4 volumes of chloroform and 0.04 volumes of water were added to compensate for the methanol added. The organic and aqueous layer were then separated by the addition of 0.2 volumes of salt solution

(0.73% NaCl). The upper, aqueous phase was pipetted off. The lower, organic phase was then dried down in a water bath at 70°C. Alcoholic KOH (10ml/g tissue) was added to the dried extract and mono, di, and triglycerides were saponified for 30 min in a water bath at 70°C. The tubes were then chilled in ice and neutralized to approximately pH 7.0 by the addition of 2.5N PCA. The solution was centrifuged at 5900g for 10 min to remove precipitated $KClO_4$. Neutralized samples were stored at -80°C. Total glycerides were determined as glycerol released after saponification. (Folch et al., 1957; Eggstein and Kuhlman, 1974).

Measurement of Metabolites

Metabolite concentrations were assayed using enzyme reactions coupled to the oxidation or reduction of pyridine nucleotides. The change in absorbance at 340nm due to the production or utilization of NADH was measured using a Gilford 252 photometer with a Beckman 2400 light source. In an assay run to completion, where only the metabolite of interest is limiting, the number of μ moles of pyridine nucleotide consumed is directly proportional to the μ moles of metabolite utilized. The number of μ moles of pyridine nucleotide used is calculated from the following formula:

$$\mu\text{mol} = \Delta\text{OD} \times \text{vol} / \epsilon$$

where ΔOD = change in absorbance of the pyridine nucleotide
at 340 nm. (final OD - initial OD)

vol = total volume of the cuvette

ϵ = micromolar extinction coefficient of pyridine
nucleotides at 340 nm 6.22

Knowing the dilution of each tissue sample during
acid extraction and the volume of neutralized extract
added to the cuvette the concentration of the metabolite
in $\mu\text{mol}/\text{gram wet wt.}$ can be determined from the formula:

$$\mu\text{mol/g wet wt.} = \mu\text{mol} \times \frac{1000 \mu\text{l}}{V_4} \times \frac{V_2 + V_3}{V_2} \times \frac{V_1 + W}{W}$$

where:

V_4 = volume of sample in the cuvette in μl

V_3 = volume of KOH used to neutralize the acid
supernatant

V_2 = volume of acid supernatant recovered

V_1 = volume of PCA used to homogenize the tissue

W = tissue weight

Each sample was run in duplicate. Standards of
known concentration were run to verify the accuracy of the
assay and blank cuvettes were run so that spurious changes
in absorbance could be subtracted from the experimental
value.

Metabolite Assays

Alanine

Glutamate pyruvate transaminase (GPT, 2.0 U) was added to a cuvette containing buffer (Tris-HCl, 100 mM, pH 8.0), NADH (100 μM), α -ketoglutarate (2 mM), lactate dehydrogenase (LDH, 0.5 U), and neutralized extract (Lowry and Passonneau, 1972).

Aspartate

Glutamate oxaloacetate transaminase (GOT, 2.0 U) was added to a cuvette containing buffer (imidazole - HCl 100 mM, pH 7.0), NADH (200 μM), α -ketoglutarate (2 mM), malate dehydrogenase (MDH, 2.0 U) and neutralized extract (Lowry and Passonneau, 1972).

Glucose

Hexokinase (HK, 0.1 U) was added to a cuvette containing buffer (Tris-HCl, 100 mM, pH 8.0), NAD (200 μM), MgSO_4 (1 mM),

ATP (1 mM), glucose-6-phosphate dehydrogenase (G-6-PDH, 0.3 U) and neutralized extract (Keppler and Decker, 1974).

Glycerol

Glycerol kinase (GK, 0.4 U) was added to a cuvette containing buffer (Tris-HCl, 100 mM, pH 7.5), NADH (200 μ M), PEP (500 μ M), MgCl_2 (1 mM), ATP (1 mM), lactate dehydrogenase (LDH, 0.4 U), pyruvate kinase (PK, 0.3 U) and neutralized extract (Eggstein and Kuhlmann, 1974).

Malate

Malate dehydrogenase (MDH, 3.5 U) was added to a cuvette containing buffer (glycine, 100 mM, pH 9.5), NAD^+ (200 μ M) glutamate (400 μ M) and glutamate oxaloacetate transaminase (GOT, 0.9 U) and neutralized extract (Lowry and Passonneau, 1972).

Strombine/Alanopine

Strombine/alanopine dehydrogenase (SDH/ADH, 1.0 U) was added to a cuvette containing buffer (hydrazine-KOH 300 mM, pH 9.0), NAD^+ (2 mM) and neutralized extract (Storey et al., 1982).

Succinate

Succinyl thiokinase (STK, 0.09 U) was added to a cuvette containing buffer (imidazole-HCl 100 mM, pH 7.0), NADH (200 μM), MgSO_4 (2 mM), KCl (75 mM), ATP (100 μM), CoA (300 μM), PEP (300 μM), lactate dehydrogenase (LDH, 0.4 U) pyruvate kinase (PK 0.3 U) and neutralized extract (Williamson and Corkey, 1969).

Statistics

Results are presented as mean $\pm$ standard error of the mean (s.e.m.). Statistical significance was assessed by the Student's t-test (Mendenhall, 1971).

Reaction conditions for the metabolite assays were designed by using optimal coupling enzyme activities and high co-substrate concentrations to drive reactions essentially to completion, utilizing > 99% of the

metabolite being assayed (Lowry and Passoneau, 1972). This was confirmed for each assay mixture by measuring a standard concentration of the substrate and confirming that it agrees with the known amount of substrate added to the cuvette. This confirmed that each reaction was run essentially to completion.

Standard curves were run for each metabolite as in Materials and Methods. Standard curves were used to calculate concentrations of metabolites in the coupled enzyme assays.

RESULTS

Long Term Temperature Acclimation and Anoxia

To observe temperature effects on anaerobic metabolism groups of clams were first acclimated for three weeks to 25°, 15°, and 5°C. Acclimated animals were then exposed to anoxia stress for times of up to 96h. The acclimation period was chosen to be long enough to allow animals to fully adjust metabolically to the three temperature regimes: this choice was based on previous studies of marine bivalves (Widdows and Bayne, 1971). Indeed, as will be discussed later, the results of this study differ markedly from those of the second study (acute temperature change and anoxia) in a manner suggesting that the animals in the present study were fully acclimated to each temperature. This investigation of anaerobic metabolism at three different temperatures may be applicable to the natural environment in that the animals must adapt to fairly wide changes in seasonal temperatures and yet must still endure daily periods of anaerobiosis.

Control

During the three week acclimation period the animals appeared to remain relatively quiescent with no observable digging activity. To assess the metabolic state of the animals at each acclimation temperature, the control groups were assayed for fuel levels. (Table 1). Glycogen, lipid, and protein in the tissues of the animals were not

Table 1: Levels of Protein, Glycogen and Lipid in tissues of Control *M. mercenaria*. Values are means $\pm$ s.e.m. for n=5 animals. Average tissue weights from n = 3 animals given as gram wet weight $\pm$ s.e.m.

Tissue		Protein mg/g.w.w.	Glycogen μ mol/g.w.w.	Lipid μ mol/g.w.w.
Mantle 0.36 ± 0.03	25°	7.6 $\pm$ 0.4	143.7 $\pm$ 4.0	3.0 $\pm$ 0.3
	15°	8.0 $\pm$ 1.3	129 $\pm$ 6.1	2.7 $\pm$ 0.6
	5°	8.5 $\pm$ 0.6	114.5 $\pm$ 29.9	2.9 $\pm$ 0.4
Gill 0.61 ± 0.10	25°	7.5 $\pm$ 0.5	26.9 $\pm$ 1.8*	3.2 $\pm$ 0.4
	15°	8.7 $\pm$ 0.4	45.6 $\pm$ 3.6	2.3 $\pm$ 0.4
	5°	8.4 $\pm$ 0.5	28.1 $\pm$ 4.2*	3.3 $\pm$ 0.4
Adductor 0.83 ± 0.17	25°	19.1 $\pm$ 1.3	75 $\pm$ 6.0	1.8 $\pm$ 0.3
	15°	21.6 $\pm$ 0.5	98.8 $\pm$ 7.4	2.0 $\pm$ 0.2
	5°	24.9 $\pm$ 0.2	74.4 $\pm$ 12.1	2.1 $\pm$ 0.3
Total Animal Weight (minus shell)				
4.46				
± 0.95				

* - metabolite levels significantly lower than that in the tissues of animals held at 15°C $p < 0.05$

apparently depleted at any of the three temperatures. This implies that none of the animals were experiencing an energy stress which would have resulted in decreased substrate reserves. Aerobic metabolism appears to be under no stress after three weeks of acclimation. Tissue differences in fuel reserves were noted. Glycogen content was highest in mantle tissue, intermediate in adductor muscle, and lowest in gill. High glycogen content in mantle is typical in marine molluscs (Zurburg, 1981) consistent with the reproductive role of the tissue. Glyceride reserves were similar in both gill and mantle, but overall about one third lower in adductor muscle. Adductor, however, had the highest protein content undoubtedly due to the content of muscle protein.

Levels of the anaerobic metabolites aspartate, alanine, and succinate (Fig. 3,4,5) were also assayed in the control animals. For the animals acclimated to 25° and 15°C the tissue levels of the metabolites were similar in all the tissues of both groups. Low succinate levels suggest little anaerobic activity within these animals. However the tissues from control animals acclimated to 5°C had alanine and succinate levels significantly higher than the levels found in tissues from animals held at warmer temperatures, while aspartate reserves of these animals were significantly lower. The increased levels of anaerobic end products (alanine and succinate) and the reduced fuel reserves (aspartate) suggests that animals held

at 5°C may be partially dependent upon anaerobic metabolism for energy production.

Anoxia at 15°C

When considering the anaerobic metabolism of the clams held at 15°C it was noted that the results closely paralleled a previous study on the same species (Korycan and Storey, 1983).

Mantle (Fig. 3, Table 2): This tissue displayed the highest reserves of glycogen in the control animals, and showed one of the highest tissue consumptions of glycogen during anoxia, 35 $\mu\text{mol/g}$ (expressed as glucose units). Aspartate use was greatest between 2 and 24h, 1.2 $\mu\text{mol/g}$, with no significant alteration seen over the remainder of the time course.

Unlike the substrates, which changed the most during the early stages of anoxia, alanine and succinate accumulated slowly over the initial 24h of anoxia. Accumulation of end products accelerated over the remaining 72h with a final ratio of succinate to alanine production of 3:1 after 96h of anoxia. Up to 24h of anoxia, succinate accumulation approximately equalled the decrease in aspartate reserves suggesting a direct use of aspartate as the substrate for succinate synthesis. However after the initial 24h of anoxia succinate production continued while no further aspartate consumption was seen indicating the need for an

Figure 3 The changes in concentration of succinate, alanine, aspartate, and glycogen in mantle of M. mercenaria during 96h of anoxia and the subsequent recovery period. Results are expressed as means $\pm$ s.e.m. for n=5 animals at each point.

o = 25°C

• = 15°C

▯ = 5°C

Figure 4 The changes in concentration of succinate, alanine, aspartate, and glycogen in gill of M. mercenaria during 96h of anoxia and the subsequent recovery period. Results are expressed as means $\pm$ s.e.m. for n=5 animals at each point.

◦ = 25°C

• = 15°C

◻ = 5°C

Figure 5 The changes in concentration of succinate, alanine, aspartate, and glycogen in adductor of M. mercenaria during 96h of anoxia and the subsequent recovery period. Results are expressed as means $\pm$ s.e.m. for n=5 animals at each point.

○ = 25°C

● = 15°C

◻ = 5°C

Table 2: Changes in the tissue levels of metabolites, 96h minus 0h, during ~~anoxia~~. Statistically significant changes were evaluated using the Student's t-test, two-tailed (Mendenhall, 1971). Values are expressed as mol/g.w.w. and calculated from Fig. 3, 4, and 5. n.s.=not significantly different.

Tissue		Glycogen	Aspartate	Succinate	Alanine
Mantle	25°	- 96.9 p<0.01	- 2.3 p<0.02	+17.4 p<0.01	+ 0.21 n.s.
	15°	- 35.5 p<0.01	- 1.2 p<0.1	+ 8.8 p<0.01	+ 3.1 p<0.01
	5°	- 30.5 n.s.	-0.37 n.s.	+ 4.2 p<0.1	+ 0.79 n.s.
Gill	25°	- 25.1 p<0.01	- 1.1 p<0.02	+16.3 p<0.01	+ 1.49 p<0.01
	15°	- 27.4 p<0.01	- 1.8 p<0.01	+ 9.2 p<0.01	+ 1.99 p<0.01
	5°	- 27.0 p<0.01	-0.02 n.s.	+ 3.9 p<0.1	+ 0.46 n.s.
Adductor	25°	- 51.6 p<0.01	- 4.9 p<0.02	+ 9.6 p<0.01	+ 0.39 n.s.
	15°	- 38.4 p<0.01	- 7.1 p<0.01	+ 4.3 p<0.02	+ 6.54 p<0.01
	5°	- 28.5 p<0.01	-0.03 n.s.	+ 0.7 n.s.	+ 0.83 n.s.

alternate substrate for succinate synthesis. This is apparently glycogen (Zurberg and Kluytman, 1980) with the PEP produced from glycogen being consumed by PEPCK to feed succinate production (Fig. 1). A partitioning of carbon at the PEP branchpoint must occur as both succinate and alanine accumulation continued simultaneously in mantle.

Upon return to aerobic conditions all metabolite levels rapidly recovered, reaching control levels within 24h.

Gill (Fig. 4, Table 2): Gill tissue from animals held at 15°C showed an overall reduced level of substrate consumption compared to the mantle. Glycogen consumption was 27 $\mu\text{mol/g}$, about one third less than that seen in the mantle. Aspartate use, however, was comparable to that of mantle, 1.8 $\mu\text{mol/g}$ over the 96h.

As in the mantle, succinate accumulation exceeded aspartate consumption, 9.2 $\mu\text{mol/g}$. Therefore, succinate is apparently derived from both aspartate and glycogen catabolism. The steady accumulation of alanine throughout anoxia, as in mantle, indicated that PEP is apparently being directed into both end products simultaneously. The ratio of succinate to alanine production was 4.6:1 after 96h of anoxia.

As in mantle there was a rapid recovery of metabolite levels reaching control levels within 24h of the return to normoxic conditions.

Adductor (Fig. 5, Table 2): The adductor muscle of clams held at 15°C had the highest level of aspartate reserves in the control group. Anaerobic aspartate consumption, 7.1 $\mu\text{mol/g}$, was greater than either the gill or mantle. Glycogen consumption was similar to that in the mantle, 38.4 $\mu\text{mol/g}$.

Overall, succinate accumulation, 4.3 $\mu\text{mol/g}$, was less than the level of aspartate consumption. Therefore it appears likely that this tissue does not require glucosyl carbon for succinate synthesis since aspartate use is sufficient to account for all succinate produces. Because glycogen does not appear to enter the succinate path it is presumed that it is being channelled solely into alanine accumulation. This leads to a high alanine accumulation, 6.5 $\mu\text{mol/g}$, in the adductor and yields a succinate to alanine ratio of 0.6:1, much lower than that seen in either gill or mantle tissues. The pattern of anaerobic metabolism seen in the adductor appears, therefore consistent with the "glucose $\rightarrow$ alanine" and "aspartate $\rightarrow$ succinate" pathways. This metabolic pattern has been noted for the adductor muscle in previous studies (de Zwaan *et al.*, 1983; Korycan and Storey, 1983).

On return to aerobic conditions all metabolite levels returned to control levels within 24h.

Summary: As mentioned earlier the anaerobic metabolism of clams held at 15°C closely resembles the overall pattern

found by Korycan and Storey (1983). Mantle showed the greatest depletion of glycogen reserves, fueling both succinate and alanine production with succinate as the primary end product. Gill behaved in a similar manner. In the adductor muscle, however, glycogen use was low and appeared to fuel alanine synthesis only. Aspartate use was highest in the adductor and this appeared to account for all succinate formed.

The metabolism of the clam at 15°C can be used as a standard against which to compare the results of anoxia at 5° or 25°C. This would allow any temperature specific changes in metabolism to be identified and described. Two possible results might be expected from animals acclimated to 25° a) no difference in the amount or pattern of anaerobic end products accumulated; this would follow if metabolic rate was independent of temperature, or b) a two fold increase in the output of end products; this would follow if typical Q_{10} effects applied to the anaerobic metabolism of molluscs.

Anoxia at 25°C

The anaerobic metabolism of clams acclimated long term to 25°C did demonstrate differences from that of animals held at 15°C. However it was not only the rate of metabolism which was affected.

Mantle (Fig. 3, Table 2): As was noted for animals held at 15°C the mantle at 25°C had the greatest glycogen reserves of the three tissues examined. Mantle showed a total glycogen consumption of 96.9 $\mu\text{mol/g}$ over the 96h of anoxia, a 2.7 fold increase over the amount used in the tissue at 15°C. In addition aspartate consumption, 2.3 $\mu\text{mol/g}$, was double the level found in the mantle at 15°C.

Mantle tissue at 25°C showed no significant accumulation of alanine over the course of anoxia. Succinate accumulation, however, was high with a net accumulation of 17 $\mu\text{mol/g}$. It appears therefore that at the higher temperature animals preferentially channel glucosyl carbon into succinate production with little or no use of the "glucose $\rightarrow$ alanine" pathway.

On return to aerobic conditions the metabolites all return to control levels within 24h of reimmersion.

Gill (Fig. 4, Table 2): The gill tissue from clams acclimated to 25°C reacted to anoxia in the same manner as the tissue from the 15°C group. Overall glycogen use was 25 $\mu\text{mol/g}$, similar to that at 15°C. Aspartate use, at 1.1 $\mu\text{mol/g}$, was also comparable between the 15°C and 25°C groups.

However, like the mantle, the gill from animals held at 25°C had a much higher succinate production, 16 $\mu\text{mol/g}$, than was found in the gill tissue of the animals held at 15°C. The ratio of succinate to alanine production was

11:1, more than double the ratio found in the gill tissue of clams at 15°C. Again, as in mantle, the tissue from animals acclimated to 25°C favored succinate production more than clams at 15°C. Carbon for succinate synthesis appears to derive largely from glucosyl carbon.

As in the mantle, all metabolites returned to control levels within 24h of return to aerobic conditions.

Adductor (Fig. 5, Table 2): Consumption of glycogen in the anoxic adductor muscle was 51.6 $\mu\text{mol/g}$, about twice that found at 15°C. Aspartate use was 4.9 $\mu\text{mol/g}$ higher than the consumption found in the other tissues of the 25°C group. Fuel use by the adductor muscle was, therefore, qualitatively similar between the 15°C and 25°C groups.

However the pattern of alanine accumulation in the adductor muscle was different between the two groups. The muscle from animals held at 15°C produced alanine steadily throughout the time course of anoxia. The adductor from clams at 25°C, however, showed a short-term alanine accumulation which was discontinued after 24h of anoxia.

Adductor from animals at 25°C also showed a high net succinate accumulation, 9.5 $\mu\text{mol/g}$, which exceeded aspartate catabolism. These results suggest an altered pattern of anaerobic metabolism in adductor at 25° compared to that at 15°C. Aspartate is apparently no longer the only substrate for succinate synthesis. It appears that the adductor at 25°C has activated the "glucose→succinate" path which was not

operative in the muscle of 15°C. This redirection of glucosyl carbon resulted in increased succinate levels, compared to the adductor at 15°C, and no significant alanine accumulation.

Return to aerobic conditions resulted in metabolite levels reaching control levels within 24h.

Summary: The overall rate of anaerobic metabolism in the clam acclimated to 25°C appears to be higher than the group at 15°C. The amount of glycogen catabolized in mantle and adductor was double that used by animals held at 15°C. The amount of succinate formed in all tissues was double the amount found in 15°C animals. Alanine levels, however, were not higher overall at 25°C. This may be because alanine is an end product only in the early hours of anoxia. This is due to the more complete redirection of carbon flow away from alanine and towards succinate synthesis at 25°C. This reduced alanine synthesis and increased succinate synthesis results in a higher succinate to alanine ratio in all tissues at 25°C (18:1 and 11:1 versus 3:1 and 4.5:1 at 15 C for mantle and gill respectively).

In the adductor muscle acclimation to 25°C resulted not only in a higher ratio of succinate to alanine production, due to increased succinate formation, but also in an alteration in the metabolic pathway used. Glycogen catabolism was apparently directed to both the succinate and alanine pathways, whereas at 15°C glycogen apparently entered only the alanine pathway. After the initial 24h of anoxia, at

25°C, all glycogen entered the succinate path with no further alanine production.

Anoxia at 5°C

The major factor bearing on the anaerobic metabolism of clams acclimated to 5°C was the apparent reliance upon anaerobic pathway during the aerobic, acclimation period. Thus animals entered anoxia with depleted aspartate reserves and high initial levels of succinate and alanine. The already depleted aspartate reserves limits the metabolic options during anoxia.

Mantle (Fig. 3, Table 2): Overall rate of carbon flux, as evidenced by endproduct accumulation, was much lower than that seen in the mantle tissue of animals at 15°C. During the initial 24h of anoxia a small amount of aspartate consumption was noted, 0.5 $\mu\text{mol/g}$ which corresponded with a similar increase in succinate. There was a simultaneous increase in alanine levels of 0.5 $\mu\text{mol/g}$, which suggests that some glycogen had been catabolized. After 24h of anoxia, alanine accumulation and aspartate consumption ceased and overall levels of alanine and aspartate after 96h of anoxia were not significantly different from those of controls. Succinate levels climbed by 4.0 $\mu\text{mol/g}$ over the remaining 72h apparently as a result of glycogen catabolism.

On return to aerobic conditions, tissue levels of succinate and glycogen returned to control levels within

24h. However aspartate and alanine levels which only had minor changes in anoxia, showed no significant change.

Gill (Fig. 4, Table 2): During the initial 24h of anoxia the gill tissue from clams acclimated to 5°C had a high rate of glycogen consumption, 16 $\mu\text{mol/g}$. However only negligible amounts of end product were formed. After the initial 24h of anoxia, glycogen use tapered off, while succinate steadily accumulated with a net increase of 3.9 $\mu\text{mol/g}$. Both aspartate and alanine levels remained constant over the anoxic period. Compared to gill tissue from animals at 15°C there was a reduced rate of metabolism, and energy production appeared to depend solely on glycogen degradation to succinate.

During the recovery period, glycogen and succinate returned to control levels within 24h. Alanine and aspartate are unchanged during recovery.

Adductor (Fig. 5, Table 2): The anaerobic metabolic rate of the adductor muscle at 5°C appeared to be slower than that of the muscle from animals held anoxic at 15°C. Glycogen consumption, 28.5 $\mu\text{mol/g}$, is 0.75 the amount used in the adductor at 15°C. Aspartate use was not detected over the time course of anoxia. There appeared to be a low level of end product accumulation, but this was not significant.

On return to normoxic conditions, glycogen returned to

control levels. Succinate levels also declined sharply, although it was not accumulated during anoxia. Alanine and aspartate levels remained unchanged during recovery.

Summary: The overall metabolic pattern of anaerobic metabolism at 5°C uses the same pathways as 15°C animals, but appears to operate at a lower rate. The reduced carbon flux implies that acclimation to 5°C has resulted in a reduced rate of anaerobic metabolism.

Glycogen Loss and Alternate End Products

The accumulation of carbon as succinate and alanine does not always account for all of the glycogen catabolized. For example, in the most extreme case, the mantle at 25°C there were 95 $\mu\text{mol/g}$ of glycogen consumed, while end products totalled 20 $\mu\text{mol/g}$ (which is 10 $\mu\text{mol/g}$ of glycogen equivalents). Although this is a common result of anaerobic metabolism in molluscs (de Zwaan and Zandee, 1972; Korycan and Storey, 1983) an effort was made to determine if the missing carbon could be accounted for by the accumulation of alternate anaerobic end products (Table 3). However, accumulation of alanopine/strombine (less than 2.0 $\mu\text{mol/g}$) and malate (less than 1 $\mu\text{mol/g}$) contribute in only a minor way to the carbon balance. A large increase in tissue or blood glucose could also account for the glycogen loss; however no significant accumulation was noted (Table 4).

A possible fate for the lost carbon might be

excretion to the mantle fluid. It is known that clams do lose carbon from their tissues to the surrounding fluids. In M. mercenaria succinate accumulates up to 35 $\mu\text{mol/ml}$ in the mantle fluid after 96h of anoxia (Korycan and Storey, 1983). This results in 140 μmoles of succinate in the average 4 ml of mantle fluid. Tissue mass of the soft tissues, mantle and gill, is much less than a gram, while the adductor averages one gram. Therefore the total loss of carbon from gill, mantle, and adductor is balanced by the accumulation of succinate in the mantle fluid.

Table 3: Levels of alternative anaerobic end products in the tissues of *M. mercenaria* after 96h of anoxia. Values are means $\pm$ s.e.m. for n=5 animals and expressed as $\mu\text{mol/g.w.w.}$

Tissue		Malate	Strombine/Alanopine
Mantle	25°	0.35 $\pm$ 0.07	0.70 $\pm$ 0.06
	15°	0.18 $\pm$ 0.01	0.60 $\pm$ 0.08
	5°	0.10 $\pm$ 0.02	0.61 $\pm$ 0.04
Gill	25°	0.36 $\pm$ 0.05	1.71 $\pm$ 0.28
	15°	0.30 $\pm$ 0.03	0.80 $\pm$ 0.22
	5°	0.32 $\pm$ 0.04	0.73 $\pm$ 0.04
Adductor	25°	0.37 $\pm$ 0.02	1.0 $\pm$ 0.16
	15°	0.50 $\pm$ 0.04	1.6 $\pm$ 0.36
	5°	0.40 $\pm$ 0.01	1.3 $\pm$ 0.22

Table 4: Glucose levels in tissues and blood samples of *M. mercenaria*. Tissue samples are in $\mu\text{mol/g}$ wet weight, means $\pm$ s.e.m. of $n=5$ animals. Blood samples of $n=5$ animals are pooled and given as $\mu\text{mol/ml}$.

Tissue		Control	24h	Anoxia	96h	Recovery 6h
Gill	25°	0.28 $\pm$ 0.04	0.28 $\pm$ 0.03	0.37 $\pm$ 0.08	0.171 $\pm$ 0.09	
	15°	0.16 $\pm$ 0.04	0.38 $\pm$ 0.05	0.55 $\pm$ 0.11	0.25 $\pm$ 0.08	
	5°	0.12 $\pm$ 0.03	0.39 $\pm$ 0.07	0.38 $\pm$ 0.08	0.19 $\pm$ 0.06	
Mantle	25°	0.30 $\pm$ 0.03	0.23 $\pm$ 0.01	0.22 $\pm$ 0.04	0.14 $\pm$ 0.01	
	15°	0.26 $\pm$ 0.03	0.25 $\pm$ 0.03	0.28 $\pm$ 0.03	0.18 $\pm$ 0.01	
	5°	0.14 $\pm$ 0.02	0.29 $\pm$ 0.03	0.25 $\pm$ 0.04	0.16 $\pm$ 0.02	
Adductor	25°	0.24 $\pm$ 0.05	0.09 $\pm$ 0.02	0.18 $\pm$ 0.04	0.05 $\pm$ 0.01	
	15°	0.25 $\pm$ 0.02	0.21 $\pm$ 0.03	0.14 $\pm$ 0.02	0.08 $\pm$ 0.02	
	5°	0.16 $\pm$ 0.02	0.17 $\pm$ 0.02	0.16 $\pm$ 0.02	0.12 $\pm$ 0.03	
Blood	25°	0.35	0.64	0.58	0.63	
	15°	0.38	0.38	0.51	0.36	
	5°	0.20	0.17	0.19	0.23	

Acute Temperature Change and Anoxia

In the previous study, animals were acclimated for three weeks to each temperature regime. In the present study the effects of acute temperature change were examined. Animals were first acclimated to 15°C for three weeks. Anoxia and an acute temperature change to 5° or 25°C were then initiated simultaneously. Such a temperature shock accompanying the onset of anoxia mimics the rapid temperature fluctuations which can occur when an intertidal animal is exposed to air at low tide during the summer or winter. Because these clams were exposed to both a temperature and anoxic stress, a parallel set of animals were monitored to test the effects of the temperature shock alone. Groups of clams were switched to 25° or 5°C and maintained in aerobic seawater for the same 96h time period as the anoxic animals. The results of this experiment can be compared to the previous acclimation study to determine the effects of long term versus acute temperature fluctuation on anaerobic metabolism.

Control Animals

The control levels of glycogen, aspartate, succinate, and alanine (Fig. 6,7,8) were similar to those found in the 15°C control animals of the previous experiment and were also in good agreement with the levels of metabolites found in M. mercenaria tissues by Korycan and Storey (1983). With regard to anaerobic fuels the highest glycogen

levels were found in the mantle; aspartate reserves were greatest in the adductor muscle. Levels of the end product succinate were relatively low in all tissues. Alanine levels were also low in the gill and mantle, however the adductor had higher levels. This however, is typical of the higher free amino acid pool found in muscle compared to soft tissue of marine molluscs (Zandee, et al., 1980b). Glucose levels in the control clams were also low, less than $0.2 \mu\text{mol/g}$, in all tissues. Tissue glucose levels increased during 96h of anoxia at 15° and 25°C (Table 5); levels never exceeded $0.9 \mu\text{mol/g}$ however.

Anoxia at 15°C

The changes in metabolites after 96h of anoxia at 15°C were similar to the pattern found in the previous acclimation study. This was as expected because both groups of animals experienced the same thermal and anoxic conditions.

Mantle (Fig. 6): Glycogen use was, again, highest in the mantle tissue with a decrease of $44 \mu\text{mol/g}$ after 96h of anoxia. A limited aspartate utilization was noted and could not account for the total succinate accumulation of $15 \mu\text{mol/g}$. This suggests an alternate carbon source, undoubtedly glycogen. Alanine levels showed no significant change over the 96h. As in mantle in the long term acclimation study the basic metabolic pathway "glucose—succinate" was present.

Table 5: Tissue glucose levels during anoxia and recovery in M. mercenaria. Values are means $\pm$ s.e.m. for n=5 animals and expressed as $\mu\text{mol/g.w.w.}$

Tissue		Control	96h Anoxia	24h Recovery
Mantle	25°	-	0.87 $\pm$ 0.02	0.39 $\pm$ 0.11
	15°	0.18 $\pm$ 0.2	0.64 $\pm$ 0.08	0.40 $\pm$ 0.10
	5°	-	0.15 $\pm$ 0.05	0.16 $\pm$ 0.02
Gill	25°	-	0.58 $\pm$ 0.05	0.35 $\pm$ 0.13
	15°	0.15 $\pm$ 0.02	0.41 $\pm$ 0.08	0.22 $\pm$ 0.04
	5°	-	0.04 $\pm$ 0.01	0.12 $\pm$ 0.02
Adductor	25°	-	0.86 $\pm$ 0.15	0.73 $\pm$ 0.13
	15°	0.18 $\pm$ 0.01	0.49 $\pm$ 0.03	0.24 $\pm$ 0.06
	5°	-	0.11 $\pm$ 0.02	0.14 $\pm$ 0.01

Glycogen and succinate levels showed a recovery upon return to aerobic conditions.

Gill (Fig. 7): The gill of animals exposed to 96h of anoxia at 15°C showed the same metabolic response as the mantle. Compared to the mantle, glycogen use was reduced to 8.0 $\mu\text{mol/g}$. Aspartate and alanine levels were not substantially changed during anoxia.

On return to aerobic conditions there was a recovery of all metabolites within 24h.

Adductor (Fig. 8): The adductor muscle had the highest level of aspartate use, 7.0 $\mu\text{mol/g}$ after 96h of anoxia. Glycogen use, 14.6 $\mu\text{mol/g}$, was comparable to that found in the gill and much lower than that found in the mantle. Succinate accumulation, 8.0 $\mu\text{mol/g}$, closely matched the amount of aspartate utilized. This suggests that aspartate was the sole substrate of the succinate pathway, with no significant glycogen contribution. Glycogen is apparently directed solely into alanine production with an increase of 6.2 $\mu\text{mol/g}$ noted.

On return to aerobic conditions all metabolites, except aspartate, recovered towards control levels. Aspartate did not show any recovery during the first 24h.

Summary: The pattern of anaerobic metabolism found in the clams held at 15°C closely matched the pattern of the

15°C group in the previous long term temperature acclimation study. This was expected as both groups experienced the same environmental conditions. The tissue specific patterns of metabolism were 1) high levels of glycogen catabolism in the mantle 2) production of succinate in both gill and mantle as the primary end product via the "glucose-succinate" pathway 3) succinate synthesis from aspartate in the adductor muscle with glycogen catabolized only to alanine. Therefore the techniques of acclimation and anoxia are relatively reproducible, yielding similar results in both 15°C groups. This confirms the validity of comparing the two studies.

Anoxia at 25°C

When comparing the results of the animals placed in anoxia with an acute temperature change to 25°C to animals held anoxic at 15°C, the most obvious change was the increased carbon flux of the animals at the warmer temperature. Within every tissue fuel consumption and end product accumulation was higher in animals moved to 25°C.

Mantle (Fig. 6): Anaerobic use of both glycogen, 48 $\mu\text{mol/g}$, and aspartate, 1.6 $\mu\text{mol/g}$, was increased above the level found in the mantle of animals at 15°C. The high level of succinate accumulation, 40 $\mu\text{mol/g}$, suggests that glycosyl carbon is required as a substrate. This is the same pathway found in the mantle tissues at 15°C and in the mantle at 25°C in the previous experiment. However, a difference

was noted in that the mantle from animals at 25°C in the acute study had a greater alanine synthesis, 7.8 $\mu\text{mol/g}$, than the mantle at 25°C in the acclimation study. This results in a lower ratio of succinate to alanine production, 5:1, in the experiment compared to 18:1 for the mantle at 25°C in the previous experiment. This suggests that animals exposed to the acute temperature stress have a more balanced partitioning of carbon at the PEP branchpoint, compared to animals subjected to long term acclimation.

During recovery, when anoxic animals were returned to water at 15°C, succinate showed a decrease in the 24h recovery period. No other metabolite showed significant changes.

Data for mantle tissue of animals exposed only to temperature stress, placed in normoxic water at 25°, is also shown on Fig. 6. Mantle under these conditions exhibited an anaerobic response. However the tissue levels of end products rose only to one half of the level found in animals exposed to both temperature shock and anoxia.

Gill (Fig. 7): The gill tissues of animals switched to 25°C at the onset of anoxia had a similar metabolic pattern as the mantle. Succinate accumulation, 40 $\mu\text{mol/g}$, was in excess of aspartate use indicating that the "glucose→succinate" pathway is functioning. Similar to the mantle tissue there was a partitioning of carbon at the PEP branchpoint resulting in an alanine accumulation of

8.0 $\mu\text{mol/g}$, and a ratio of succinate to alanine production of 5:1. This is the same ratio found in the mantle. The overall carbon flux was higher than that in the gill at 15°C. When comparing the acute and long term acclimation studies, a more balanced succinate to alanine ratio, 5:1 versus 11:1 respectively, was found, again similar to the mantle at 25°C.

However recovery of metabolite levels was more complete in the gill tissue. Within 24h of return to aerobic seawater, glycogen, succinate, and alanine had recovered to one half of the control value, aspartate was fully recovered to the control value.

Gill tissue from animals exposed only to the temperature shock, normoxic water at 25°C, had an anaerobic response similar to that seen in the mantle. Succinate and alanine accumulated to levels about one half of those found in animals exposed to temperature shock plus anoxia. Presumably this represents an early step in acclimation to the new temperature.

Adductor (Fig. 8): The adductor muscles of animals exposed to 25°C at the onset of anoxia showed significant differences from the muscle of animals held at 15°C during anoxia. The accumulation of succinate, 30 $\mu\text{mol/g}$, was far in excess of aspartate use, 8.8 $\mu\text{mol/g}$. This indicates that the muscle requires glucosyl carbon to maintain such a high level of succinate synthesis. There was also significant alanine synthesis, 7.8 $\mu\text{mol/g}$, which

indicates that PEP is partitioned into both alanine and succinate synthesis. This differed from the muscle of animals held at 15°C which used glycogen for alanine synthesis only with aspartate as the sole substrate for succinate production. This shift in anaerobic metabolism was also present in the adductor of animals acclimated in the long term to 25°C. Therefore in the adductor muscle there is an increased emphasis on succinate production during both acute and long term exposure to warmer temperature conditions.

During recovery both alanine and succinate levels recovered towards the control levels. Glycogen and aspartate levels remained constant during the initial 24h of recovery.

Adductor muscle from animals exposed only to the high temperature stress, normoxic water at 25°C, had an anaerobic response greater than that seen in the mantle or gill. Amounts of glycogen and aspartate used were also greater in adductor of these animals than in the muscles of animals exposed to both anoxia plus temperature stress. This was also true of alanine accumulation, 15 $\mu\text{mol/g}$, but not succinate production which was reduced to 11 $\mu\text{mol/g}$. This increased anaerobic energy production of the adductor muscle of clams in normoxic water at 25° may be due to increased muscle work during shell opening and closure with a high accumulation of the glycolytic end product, alanine, as a product of muscle work.

Summary: Again, as was the case with animals acclimated to 25°C for a long term, the overall rate of anaerobic metabolism appears to be higher in animals switched to anoxia at 25°C than in animals made anoxic at 15°C. All tissues showed an increased level of fuel consumption and end product formation indicating a higher anaerobic metabolic rate at 25° than at 15°C. In the adductor muscle of animals moved to 25°C there was not only an increase in carbon flux, but also a change in the pattern of anaerobic metabolism. The shift noted was to succinate production from both aspartate and glycogen catabolism.

The effect of imposing a similar thermal shock on clams without the accompanying anoxic stress also resulted in an anaerobic response. The gill and mantle showed a lower level of anaerobic activity than animals exposed to both stresses. This implies either an anaerobic energy production to supplement aerobic metabolism during temperature acclimation, or a prolonged period of shell closure as an initial response. While the muscle work of adductor from animals exposed to thermal stress resulted in increased emphasis on alanine production.

Anoxia at 5°C

The results of combining anoxia with low temperature stress appear to be a lower metabolic rate than was seen in animals held anoxic at 15°C. In all tissues the amounts of substrate consumed and end product released were lower in animals that had been switched to cooler conditions

Mantle (Fig. 6): The amounts of glycogen and aspartate used in the mantle of animals switched to anoxia at 5°C were insignificant. Succinate was the primary end product, an increase of 4.3 $\mu\text{mol/g}$, being well in excess of aspartate utilization. Therefore it appears that glycogen must be catabolized as a carbon source. The level of end product accumulation via the "glucose $\rightarrow$ succinate" pathway was the same as that found in the mantles of animals at 5°C in the previous experiment.

On return to aerobic conditions, both glycogen and succinate levels recovered towards control levels. Aspartate and alanine were relatively unchanged during both anoxia and recovery.

The mantle of animals that were exposed only to the low temperature stress, placed in normoxic water at 5°C, displayed an anaerobic response. Glycogen was utilized (14 $\mu\text{mol/g}$); however only low levels of end products accumulated compared to animals exposed to thermal plus anoxic stresses.

Gill (Fig. 7): The metabolic pattern found in the gill tissues of animals switched to anoxia at 5°C was very similar to the mantle pattern. Succinate was the primary end product with an accumulated increase of 3.7 $\mu\text{mol/g}$. The succinate was apparently derived from glycogen catabolism, 3.27 $\mu\text{mol/g}$. Both aspartate and alanine showed no significant changes after 96h of anoxia. These are very similar results to

those found in the anoxic gill of animals exposed to long term acclimation at 5°C.

During recovery both succinate and glycogen levels declined over the first 24h but control levels were not re-established within this time period.

Similar to the mantle, the gill had an anaerobic response to acute low temperature shock alone. The changes in glycogen and succinate levels during exposure to normoxic water at 5°C were less than those found in animals exposed to both temperature plus anoxic stress. This response, of both gill and mantle, may be the result of self-induced anoxia brought about by prolonged shell closure in response to the low temperature shock.

Adductor (Fig. 8): Much like the results for animals held at 15°C, in the animals switched to 5°C, the adductor had the highest tissue use of aspartate, 2.8 $\mu\text{mol/g}$. Aspartate use equalled succinate synthesis, 2.8 $\mu\text{mol/g}$, suggesting that it is the sole substrate of the succinate pathway. There was no significant alanine accumulation or glycogen consumption in the adductor at 5°C. The type of metabolism "one substrate: one end product" is found in all adductors at 15° or 5°C. However in the adductor of animals acclimated long term to 5°C the substrate is glycogen, not aspartate.

Recovery of control levels of aspartate and succinate was not complete within 24h of return to aerobic conditions

The adductor muscle of animals exposed to low temperature stress in normoxic water at 5°C, displayed an anaerobic response similar to the muscle of animals exposed to high temperature shock. This was largely seen as an emphasis on alanine as the primary end product, 4.4 $\mu\text{mol/g}$ accumulation; this differs from the muscle of animals exposed to both temperature plus anoxic stresses where succinate was the main end product. It could be speculated that this response is due to the muscular activity of shell movement. This may result in glycolytic energy production favoring alanine as the end product.

Summary: Animals switched to anoxia at 5°C appear to have a reduced rate of carbon flux compared to clams held at 15°C. This implies a reduced rate of anaerobic metabolism in response to an acute temperature drop. When animals were exposed to an acute drop in temperature only, there was also an anaerobic response. It appears that this is a result of prolonged periods of valve closure at low temperature which induce anoxic conditions within the animal. It seems that this behaviour is maintained since animals acclimated for three weeks at 5°C in the previous experiment still had signs of anaerobic activity.

During recovery from anoxia, animals moved to 25° and 5°C generally were slow in re-establishing control levels of metabolites, compared to animals in the long term acclimation study. This is probably a result of return to

15°C water causing another temperature shock. We have noted that temperature shock under aerobic conditions leads to anaerobiosis, therefore recovery is much slower than in animals which had no temperature shock.

Figure 6 The changes in concentration of succinate, alanine; aspartate, and glycogen in mantle of M. mercenaria during 96h of anoxia and the subsequent recovery period. Results are expressed as means $\pm$ s.e.m. for n=5 animals at each point.

= 25°C

= 15°C

= 5°C

Hartle

Figure 7 The changes in concentration of succinate, alanine, aspartate, and glycogen in gill of M. mercenaria during 96h of anoxia and the subsequent recovery period. Results are expressed as means $\pm$ s.e.m. for n=5 animals at each point.

= 25°C

= 15°C

= 5°C

Figure 8 The changes in concentration of succinate, alanine, aspartate, and glycogen in adductor of M. mercenaria during 96h of anoxia and the subsequent recovery period. Results are expressed as means $\pm$ s.e.m. for n=5 animals at each point.

= 25°C

= 15°C

= 5°C

ADDITION

Table 6: Q_{10} values for accumulation of succinate and use of glycogen in the tissues of M. mercenaria after 96h of anoxia.

I Temperature Acclimation Study

Tissue	Glycogen		Succinate	
	25°-15°	15°-5°	25°-15°	15°-25°
Mantle	2.7	1.2	2.0	2.0
Gill	1.0	1.0	1.9	2.8
Adductor	2.0	1.0	2.2	∞

II Acute Temperature Study

Mantle	1.1	6.0	2.6	3.4
Gill	2.0	2.6	2.8	3.8
Adductor	2.2	∞	3.7	2.8

∞ = no end product formation or fuel use at lower temperature

The studies of Newell and Pye (1970), Shirley et al. (1978) and Widdows (1976) have all produced evidence that the aerobic metabolic rate of intertidal molluscs is controlled independent of temperature fluctuation. This would appear to have great value in the intertidal environment which is subject to long term, seasonal temperature changes and daily, acute temperature fluctuations as a result of the tidal cycle. Over a physiological range of temperature the animal is presumably able to maintain the most efficient metabolic rate to suit its energy balance. However, intertidal animals may spend a large portion of their time under severely anoxic conditions depending upon anaerobic metabolism for their energy production. One could speculate that anaerobic metabolism should also show a temperature independence, although most studies to date have only investigated oxygen consumption and therefore aerobic metabolic rate. It would make sense to maintain a constant rate of anaerobic metabolism to control the rate of fuel consumption and the formation of acidic endproducts. If anaerobic metabolism were not temperature independent it could be predicted that a temperature fluctuation would result in a rapid use of fuel reserves and a high build up of acidic waste leading to a greatly shortened anaerobic tolerance. However the prediction that ~~anaerobic metabolic~~ rate, like aerobic metabolic rate, should be temperature independent is not borne out by the results of this study.

Studies of aerobic metabolic rate have the advantage

in that an easily measurable substrate, O_2 , is quantitated and O_2 use is continuous despite the substrate (lipid, protein, or carbohydrate) being catabolized. To follow anaerobic metabolism, a variety of substrates are consumed and several end products produced. Only those metabolites which are used or produced at a continuous rate throughout the time period monitored are useful as indicators of anaerobic metabolic rate. In this regard, then, the present study suggests that succinate would be the best indicator of anaerobic metabolism. In most instances succinate accumulates in a linear fashion throughout anoxia. Alanine, the other end product measured, is a poor indicator of metabolic rate. Glycogen is in most instances utilized in a continuous manner throughout anoxia and is thus suitable as an index of anaerobic metabolic rate although the very high and variable levels of glycogen in the tissues of individuals make glycogen a less effective index than succinate in some cases. Glycogen catabolism was used by de Zwaan and Zandee (1972) to measure anaerobic metabolism at 6° and 20°C in the mussel, M. edulis. Their results suggest that anaerobic metabolic rate is temperature dependent with a doubling of glycogen use at the higher temperature.

In the first study of animals exposed to long term temperature acclimation prior to anoxia, temperature dependent differences in both glycogen use and succinate accumulation were noted (Table 6). When comparing glycogen

catabolism at 25° versus 15° there is an increased consumption at the higher temperature in both mantle and adductor with Q_{10} 's of 2.7 and 2.0 respectively. Succinate production in all tissues was also temperature sensitive between 15°- 5°C and 25° - 15°C the Q_{10} values ranged from 2 to 3. This evidence strongly suggests that anaerobic metabolism in bivalve molluscs is temperature sensitive, similar to the metabolism of other ectotherms. It appears to be that the temperature dependence of anaerobic metabolism does not seriously impair the ability to tolerate anoxia in nature. Naturally, clams acclimated to low temperatures may find their reduced rate of metabolism to be advantageous. Compared to summer clams, winter animals have reduced glycogen reserves (Zandee et al., 1980b), therefore they need to maintain a lower rate of fuel use. A corollary to this is that the high glycogen reserves of summer clams are better able to sustain a high rate of anaerobic metabolism than the winter clams. Therefore a matching of anaerobic metabolic rate to the feeding cycle may be more advantageous than maintaining a metabolic rate independent of temperature.

The first study indicates there are differences in the anaerobic metabolism of clams acclimated to different temperatures. The second study indicates the same quantitative difference also to be present between groups exposed to acute temperature changes. Increases in both glycogen use and

Table 6: Q_{10} values for accumulation of succinate and use of glycogen in the tissues of M. mercenaria after 96h of anoxia.

I Temperature Acclimation Study

Tissue	Glycogen		Succinate	
	25°-15°	15°-5°	25°-15°	15°-25°
Mantle	2.7	1.2	2.0	2.0
Gill	1.0	1.0	1.9	2.8
Adductor	2.0	1.0	2.2	∞

II Acute Temperature Study

Mantle	1.1	6.0	2.6	3.4
Gill	2.0	2.6	2.8	3.8
Adductor	2.2	∞	3.7	2.8

∞ = no end product formation or fuel use at lower temperature

succinate production are observed in all tissues of animals moved from 15° to 25°C at the onset of anoxia (Table 6). The Q_{10} values for succinate ranged from 2.6 to 3.7. The opposite result, a reduced glycogen catabolism and succinate accumulation, is observed with the acute temperature change to 5°C. The Q_{10} values for succinate ranged from 2.8 to 3.8.

The clear dependence of carbon flux on temperature change (in all tissues) indicates that anaerobic metabolism is sensitive to acute temperature change. Sudden temperature change, a common phenomena in the intertidal zone, does not appear to pose a threat to bivalve survival. The temperature change would only persist during aerial exposure and is then reversed when the tide returns within 6-12 hours. This is well within the survival times of the experimental anoxia. Another point to remember is that the rate of anaerobic metabolism is generally less than 10% of the aerobic rate (Shick et al., 1983). Therefore even a doubling of the anaerobic rate represents only a small increase in absolute energy consumption. So within the environmental range of temperature there is little risk of depleting glycogen reserves.

The effects of temperature on anaerobic metabolism were not restricted to the rate of carbon flux, but were also found to influence the pathways of carbohydrate catabolism. This was most obvious in the metabolism of the

adductor muscle. When animals were held at 15°C the pathway of carbon flow in the adductor was distinctly different from that of the mantle or gill. Over the course of 96h of anoxia in the adductor, glycosyl carbon entered only the alanine pathway, while the large tissue reserve of aspartate was the sole substrate for the succinate pathway. This metabolic pattern of "one substrate→one end product" was also found in the adductor of animals at 5°C, both after long term acclimation and in the acute temperature study. Animals acclimated in the long term to 5°C, however, had low aspartate reserves resulting in "glycogen→alanine" being the major pathway. In the acute experiment 5°C animals had a large aspartate reserve and the adductor produced greater amounts of succinate. This pattern of metabolism in the adductor has been demonstrated in other species (deZwaan et al., 1983) leading to the premise that the "PEP branchpoint" is operative during anoxia in soft tissues, but not in muscle tissues.

However the animals undergoing anoxia at 25°C, in both the acute and long term study, had a different metabolic pattern in the adductor muscle. During anoxia, the adductor muscle from animals at 25°C apparently catabolizes glycogen to both alanine and succinate. It appears that at the higher temperature, the ability to partition carbon at the PEP branchpoint, in the adductor muscle, is triggered. The probable reason for this is an increased energy demand at the higher temperature. Therefore

the warmer adductor muscle not only has an increased carbon flux, but it also partitions more of the carbon into the succinate pathway with increased energy yield. This change in metabolic carbon flow is centered around the partitioning of carbon at the PEP branchpoint. It is known that pyruvate kinase is the control enzyme at this point and the regulation involves both allosteric effects and phosphorylation/dephosphorylation changes. It would therefore seem apparent that the regulation of PK also has temperature dependent components and that in this case higher temperature potentiates the inhibition of PK activity. This theory is supported by a study of PK in the adductor muscle of M. edulis (Holwerda et al., 1983). It was found that high temperature acclimation of the animal resulted in a reduced activity enzyme variant. Therefore we find that not only is the rate of anaerobic metabolism sensitive to temperature, but the actual pathway used can also be temperature dependent. The reason for the increased emphasis on succinate production at 25°C may be a result of increased energy demand. The energetic yield of the "glucose→succinate" pathway is greater than the other possible pathways, such that an increased energy demand at 25°C could preferentially switch metabolism to the most energy efficient pathway.

Therefore it could be stated that in addition to tissue specific, species specific, and time dependent differences in anaerobic metabolism of bivalves we have also shown temperature dependent differences.

It is apparent that anaerobic metabolism is significantly changed in the adductor muscle of clams at 25°C. This is also true in the gill and mantle, although the pathways are altered quantitatively but not qualitatively from the ones used by the tissues of animals held under cooler conditions. As has been discussed, the rate of carbon flux has increased in warmer animals, however in the animals acclimated long term to 25°C there is also a change in the partitioning of carbon into alanine and succinate. A consideration of the ratio of succinate to alanine production in the gill and mantle of animals at 25°C, 10:1 and 18:1 respectively, compared to the tissues of animals at 15°C, 5.0:1 and 3:1 respectively, showed that succinate was almost the exclusive end product of animals acclimated to 25°C. This is similar to the emphasis on succinate production in the adductor, although no new pathways are involved. Again it would seem that animals acclimated to 25°C were utilizing the high ATP yield of the succinate pathway to help satisfy the increased energy demand of an accelerated metabolism. This dependence on succinate production in warm acclimated animals agrees quite well with a seasonal study of anaerobic end products in M. edulis (Zandee et al., 1980b). Animals collected in the summer and subjected to 48h of anoxia had a increased accumulation of succinate and propionate compared to winter animals. The question that is now faced is whether the increased succinate production is directly triggered by high temperature acclimation or whether it is merely a consequence of increased

metabolic rate which leads to an earlier redirection of carbon at the PEP branchpoint than is found in the tissues of cooler animals.

A possible insight into this question is presented in the acute study of temperature change and anoxia. Although these animals were not acclimated to high temperatures the group at 25°C displayed an increased level of anaerobic fuel use and end product formation. These animals appear to have a higher rate of anaerobic metabolism compared to animals held under cooler conditions, which implies that long term acclimation is not required for an increase in anaerobic flux. However the tissues of animals exposed to acute temperature stress at 25°C had a more balanced accumulation of anaerobic end products compared to animals acclimated to 25°C. The ratio of succinate to alanine production in the gill and mantle both were much lower than the ratios in the tissues of animals acclimated in the long term to 25°C. When the end product level of normoxic clams under acute high temperature stress were subtracted from the end product levels of animals under thermal plus anoxic stress this ratio of succinate to alanine is still unchanged. Therefore it appears that it is the long term acclimation to 25°C, not just the increased metabolic demand which results in a metabolic shift favoring the "glucose→succinate" pathway. This shift to a more energy efficient pathway illustrates the adaptations of which bivalve anaerobic metabolism is capable. Thus two responses of anaerobic

metabolism to high temperature are apparent 1) the acute response is to increase metabolic rate 2) the long term response is to increase metabolic rate and to alter metabolic pathways to place an emphasis on the high energy yield "glucose—succinate" pathway. These two responses may be those found in nature, the first operating when short term temperature increases are encountered, the second coming into play during the warm summer months.

Another temperature dependent difference in the metabolism of the animals was noted. In the long term acclimation study, control animals at 25° and 15°C had relatively normal levels of substrates and end products. However clams acclimated to 5°C showed a significant consumption of aspartate reserves and accumulation of succinate and alanine in the tissues prior to anoxic stress. These results suggest an active anaerobic metabolism over the three weeks of acclimation even though all other environmental conditions were similar to the animals held at 15° and 25°C and animals were maintained in well aerated tanks. An anaerobic component to normoxic bivalve metabolism has also been reported in other studies (Shick et al., 1983; Hammen, 1980). Low temperature, perhaps because in nature it is associated with winter and low food availability, appears to induce clams to spend longer time periods with closed valves, and therefore in an anoxic state.

An effect of acute temperature change in stimulating valve closure to buffer the temperature shock is probably

also responsible for the observed anaerobic response of animals switched to 25° or 5°C under normoxic conditions. Thus the primary response of clams to any adverse condition (temperature stress, salinity change, low oxygen, pollution) is probably to close the valves, reduce the metabolic rate, rely on anaerobic metabolism, and, if necessary, begin to acclimate to the new conditions. The overall lower levels of anaerobic products in the normoxic animals after 96h at the new temperature suggest that an initial valve closure response had occurred and that aerobic and anaerobic metabolism has been mixed over the 96h. Results showed particularly high levels of alanine in the adductor muscle of these clams. This could arise if alanine accumulated as a product of glycolytic muscle work during valve opening and closing.

The results of the study allow us to address the original questions set out in the introduction 1) Is the rate of anaerobic metabolism temperature dependent? From the Q_{10} values for glycogen use and succinate accumulation it appears that anaerobic metabolic rate is temperature dependent. This is true whether there was an acute temperature change or whether the animals had long term acclimation to different temperatures. Due to the low rate of anaerobic activity, however, it does not appear likely that temperature limits anoxic survival under normal environmental conditions. 2) Is the ratio of end product accumulation different at different temperatures? It appears that temperature

does affect the choice of anaerobic end products. This is most obvious in the adductor muscle which has a much greater dependence on succinate production when the animal is held anoxic at 25°C. 3) Is the anaerobic metabolism of animals acclimated to different temperatures the same as that of animals subjected to acute temperature change? Both groups of animals are similar with respect to changing metabolic rate due to changing temperature. The animals acclimated to 25°C, however, have an increased emphasis on succinate as an anaerobic end product in the gill and mantle. Animals moved to 25°C at the onset of anoxia however have a more balanced accumulation of succinate and alanine in these tissues.

The results of these studies suggest that temperature does have important affects on the anaerobic metabolism of Mercenaria mercenaria. The extremes of temperature and oxygen availability in the intertidal zone effect many species. It can be postulated that these animals also experience qualitative and quantitative changes in anaerobic carbon flux. The nature of these changes will become clear with further study.

REFERENCES

22
OF / DE

- Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
- Bullock, T.H. 1955. Compensation for temperature in the metabolism and activity of poikilotherms. *Biol. Rev.* 30: 311-342.
- Collicutt, J.M. and Hochachka, P. 1977. The Anaerobic Oyster Heart: Coupling of glucose and aspartate fermentation. *J. Comp. Physiol.* 115: 147-157.
- Crenshaw, M.A. and Neff, J.M. 1969. Decalcification of the mantle-shell interface in molluscs. *Am. Zoologist.* 9: 881-885.
- Eggstein, M. and Kuhlmann, E. 1974. Triglycerides and Glycerol: Determination after alkaline hydrolysis. In. Methods of Enzymatic Analysis. 2nd ed. (ed. H.U. Bergmeyer) Academic Press, New York. 1825-1831.
- Folch, J., Lees, M., and Sloan Stanley, G.H. 1957. A simple method for the isolation and purification of total lipids from animals tissues. *J. Biol. Chem.* 226: 497-509.
- Hammen, C.S. 1980. Total energy metabolism of marine bivalve mollusks in anaerobic and aerobic states. *Comp. Biochem. Physiol.* 67 A: 617-621.
- Hochachka, P.W. and Mustafa, T. 1972. Invertebrate Facultative Anaerobiosis. *Science.* 178: 1056-1060.
- Hochachka, P.W., Fields, J. and Mustafa, T. 1973. Animal Life Without Oxygen: Basic biochemical mechanisms. *Am.*

Zoologist. 13: 543-555.

Holwerda, D.A., Veenhof, P.R., Heutgam, H.A. van, and Zandee, D.I. 1983. Modification of mussel pyruvate kinase during anaerobiosis and after temperature acclimation. *Mol. Physiol.* 3: 225-234.

Keppler, D. and Decker, K. 1974. Glycogen: Determination with Amyloglucosidase. In. Methods of Enzymatic Analysis. 2nd ed. (ed. H.U. Bergmeyer) Academic Press, New York. 1127-1131.

Kluytmans, J.H., de Bont, A.M.T., Kruitgagen, E.C., Ravestein, H.J.L., and Veenhof, P.R. 1983. Anaerobic capacities and anaerobic energy production of some Mediterranean bivalves. *Comp. Biochem. Physiol.* 75B: 171-179.

Korycan, S.A. and Storey, K.B. 1983. Organ-specific metabolism during anoxia and recovery from anoxia in the cherry-stone clam, Mercenaria mercenaria. *Can. J. Zool.* 61: 2674-2681.

Lowry, O.H. and Passonneau, J.V. 1972. A Flexible System of Enzymatic Analysis. Academic Press, New York. 147-217.

Mendenhall, W. 1971. Introduction to Probability and Statistics. 3rd ed. Duxbury Press, Belmont, Calif..

Newell, R.C. and Pye, V.L. 1970. Seasonal changes in the effect of temperature on the oxygen consumption of the winkle Littorina littorea (L.) and the mussel Mytilus edulis (L.). *Comp. Biochem. Physiol.* 34: 367-383.

Newell, R.C. and Pye, V.L. 1971. Quantitative aspects of the relationship between metabolism and temperature in the winkle Littorina littorea (L.). *Comp. Biochem.*

Physiol. 38: 635-650.

Plaxton, W.C. and Storey, K.B. 1984.a. Purification and properties of aerobic and anoxic forms of pyruvate kinase from red muscle tissue of the channeled whelk, Busycotypus canaliculatum. Eur. J. Biochem. inpress.

Plaxton, W.C. and Storey, K.B. 1984.b. In vivo phosphorylation of red muscle pyruvate kinase from the channeled whelk, Busycotypus canaliculatum, in response to anoxic stress. Eur. J. Biochem. in press.

Precht, H. 1958. Concepts of temperature adaptation of unchanging reaction systems of cold-blooded animals. In. Physiological Adaptation (ed. C.L. Prosser) Amer. Physiol. Soc. Washington, D.C.: 50-78.

Prosser, C.L. 1955. Physiological variations in animals. Biol. Rev. 30: 226-262.

Shick, J.M., Zwaan, A. de, and de Bont A.M.T. 1983. Anoxic metabolic rate in the mussel Mytilus edulis L. estimated by simultaneous calorimetry and biochemical analysis. Physiol. Zool. 56 (1): 56-63.

Shirley, T.C., Denoux, G.J., and Stickle, W.B. 1978. Seasonal respiration in the marsh periwinkle, Littorina irrorata. Biol. Bull. 154: 322-334.

Stokes, T.M. and Awarpa, J. 1968. Alanine and succinate as end products of glucose degradation in the clam, Rangia cuneata. Comp. Biochem. Physiol. 25: 883-892.

Storey, K.B., Miller, D.C., Plaxton, W.C., and Storey, J.M. 1982. Gas-liquid chromatography of alanopine and strombine in tissues of marine invertebrates. Anal. Biochem. 125: 50-58.

- Tribe, M.A. and Bowler, K. 1968. Temperature dependence of 'standard metabolic rate' in poikilotherms. *Comp. Biochem. Physiol.* 25: 427-436.
- Widdows, J. 1976. Physiological adaptation of Mytilus edulis to cyclic temperatures. *J. Comp. Physiol.* 105: 115-128.
- Widdows, J. and Bayne, B.L. 1971. Temperature acclimation of Mytilus edulis with reference to its energy budget. *J. mar. biol. Ass. U.K.* 51: 827-843.
- Wijsman, T.C.M. 1975. pH fluctuations in Mytilus edulis L. in relation to shell movements under aerobic and anaerobic conditions. In: *Proc. 9th Europ. Mar. Biol. Symp.* (ed. H. Barnes). Aberdeen University Press, Aberdeen 139-149.
- Williamson, J.R. and Corkey, B.E. 1969. Assays of intermediate of the citric acid cycle and related compounds by fluorometric enzyme methods. In: *Methods in Enzymology* Vol 13 (ed. J.M. Lowenstein) Academic Press. New York. 434-513.
- a) Zandee, D.I., Howerda, D. and Zwaan, A. de. 1980. In: Animals and Environmental Fitness (ed. R. Gilles), Pergamon Press, New York. 181-205.
- b) Zandee, D.I., Kluytmans, J.H., Zurburg, W. and Pieters, H. 1980. Seasonal variations in biochemical composition of Mytilus edulis with reference to energy metabolism and gametogenesis. *Neth. J. Sea Research* 14(1): 1-29.
- Zurburg, W. 1981. Environmental Influences on the Energy Metabolism in Different Organs of Mytilus Edulis L. Studio Pascal, Utrecht. PhD Thesis, State University of Utrecht.

- Zurburg, W. and Kluytmans, J. 1980. Organ specific changes in energy metabolism due to anaerobiosis in the sea mussel Mytilus edulis (L.). Comp. Biochem. Physiol. 67 B: 317-322.
- Zurburg, W. and Ebberink, E.H.M. 1981. The anaerobic energy demand of Mytilus edulis. Organ specific differences in ATP-supply processes and metabolic rates. Mol. Physiol. 1: 153-164.
- Zwaan, A. de 1977. Anaerobic energy metabolism in bivalve molluscs. Oceanogr. Mar. Biol. Ann. Rev. 15: 103-187.
- Zwaan, A. de 1983. Carbohydrate Metabolism in Bivalves. In. The Molluscs. Vol. 1 Metabolic Biochemistry and Molecular Biomechanics. (ed. P.W. Hochachka). Academic Press. Toronto. 137-175.
- Zwaan, A. de and Zandee, D.I. 1972. The utilization of glycogen and accumulation of some intermediates during anaerobiosis in Mytilus edulis L. Comp. Biochem. Physiol. 43B: 47-54.
- Zwaan, A. de, Bont, A.M.T. de, and Hemelraad, J. 1983. The role of phosphoenolpyruvate carboxykinase in the anaerobic metabolism of the sea mussel Mytilus edulis L. J. Comp. Physiol. 153: 267-274.

END

1 0 0 6 8 6

FIN